Interim Progress Report for CDFA Agreement Number 12-0128-SA

Project Title: Evaluation of Pierce's Disease Resistance Resistance in Transgenic Vitis vinifera grapevines Expressing Xylella fastidiosa Hemagglutinin Protein

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Reporting period: The results reported here are from work conducted from 10/1/15 to 3/1/16

INTRODUCTION

Note: Professor Bruce Kirkpatrick was the original PI of record on this project. This report is being prepared by David Gilchrist who has accepted the responsibility to complete the data collection on this project following the recent death of Dr. Kirkpatrick and to submit the final report after June 30, 2016.

The bacteria *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease of grapes and is spread from plant to plant by xylem feeding insects. *Xf* cell-cell attachment is an important virulence determinate in Pierce's disease as shown by previous research. Two secreted hemagglutinin (HA) genes named *Hxf*A and *Hxf*B are required for adhesion and if either is mutated, *Xf* cells no longer clump in liquid medium and the mutants form dispersed "lawns" when plated on solid PD3 medium (Guilhabert and Kirkpatrick, 2005). Both mutants are hypervirulent when mechanically inoculated into grapevines, i.e. they colonize faster, cause more severe disease symptoms and kill vines faster than wild type Xf. If either *Hxf*A or *Hxf*B is individually knocked out there is no cell-cell attachment, which suggests that both HA genes are needed for cell-cell attachment. It is clear that these proteins are very important determinants of pathogenicity and attachment in *Xf*/plant interactions. Research by others PD researchers have shown that *Hxf*s were regulated by an *Xf*-produced compound DSF (Newman et al. 2004) and that they were important factors in insect transmission (Killiny and Almeida, 2009). The *Xf* HAs essential acts as a "molecular glue" that is essential for cell-cell attachment and likely plays a role in *Xf* attachment to xylem cell walls and contributes to the formation of *Xf* biofilms.

Greenhouse pathogenicity evaluations of HA-transgenic lines showed that 8 independent lines had disease severity ratings in greenhouse grown vines that were considerably less in the transgenic lines than the non-transgenic controls. Three are full length HA transgenes (PGIP220-) and five are just adhesion domains 1 through 3 transgenes (SPAD1-). Permits to establish a field planting of the HA vines were obtained with the assistance of PIPRA and a field trial was established in April 2013. The vines were inoculated with Xf in spring 2014 and PD symptoms of HA-transgenics were compared to non-transgenic, Xf-inoculated control in September 2014. Vine were then pruned back to 2 buds and allowed to go through the winter; PD symptoms on the vines were rated in September 2015.

In three out of the 5 lines expressing the HA adhesion domains only, the majority of the vines showed no PD symptoms. In the 2 other adhesion domain lines the majority of the vines were dead or had severe PD symptoms on the canes. In the 3 full length HA gene construct lines the majority of all the vines were healthy with no PD symptoms. These are initially encouraging results that reflect the results of greenhouse testing and the occurrence of PD symptoms on the canes of field vines following inoculation in 2014.

OBJECTIVES

1. Secure permits to plant HA transgenic lines in the field at UCD. Plant transgenic vines in the field and train them into a traditional bilateral cordon.
2. Inoculate 4 canes on each HA-transgenic field vine with wt Xf in spring 2014. Rate PD symptoms in September 2014 on inoculated canes. Take samples for qPCR.
3. Cut back all canes to 2 buds and rate cane growth in Spring 2016 and PD symptoms in September 2016 to determine if expression of *Xf* HA in the transgenic vines retarded or prevented movement of the inoculated *Xf* into the cordons, resulting in systemic PD.

ACTIVITIES AND SUMMARY OF RESULTS TO ACCOMPLISH OBJECTIVES

Objective 1:
Approximately 50 HA-transgenic vines representing all the transgenic lines that were produced were planted in the field in April 2013 and trained as bilateral cordons (Figure 2).

Figure 2. HA-transgenic and non-transgenic control vines planted in the field. Photo August 2015.

Objective 2:
A combination of *Xf* Temecula and Stags leap strains were grown on solid PD3 medium and the cells were harvested and suspended in phosphate buffered saline to a concentration of 10^8 cells/ml. Four canes on replicates of each transgenic line were labelled and then mechanically inoculated with a 20ul drop of *Xf* cell suspension. Inoculations were done in mid-May 2014 and inoculum droplets were quickly taken up by the transpiring canes.

Table 3: PD symptom ratings of HA-transgenic grapevines

Transgenic Lines	**# Inoculated Vines**	**# of PD Rated Canes**	**Mean Plant Disease Rating** (cane ratings)
Adhesion Domain			
AD 6	3	10	**0.7** (5as0; 4as1; 2as2)
AD 7	4	15	**0.9** (7as0; 2as1; 6as2)
AD 8	5	20	**1.6** (2as0; 5as1; 12as2;
AD 10	3	10	**1.7** (1as0; 2as1; 6as2; 3as1)
AD 12	5	19	**1.2** (5as0; 5as1;9as2)
Complete HA Gene			
220-1	4	10	**1.6** (4as0; 1as1; 6as2; 1as3)
220-3	3	12	**1.3** (4as0; 1as1; 6as2; 1as3)
220-11	3	10	**0.3** (8as0; 1as1; 1as2)
Control	3	12	(13as5; 3as4; 2as3)

Note: PD symptoms of inoculated transgenic canes were rated August 2015. Symptom ratings of individual canes were as follows:
0 is no symptoms of PD, i.e. no scorched leaves on cane;
1 is 2 to <10% scorched leaves on cane;
2 is >10% to <75% scorched leaves on cane;
3 is all leaves showing PD scorch symptoms, no cane dieback observed;
4 is cane dieback, cane still alive; and
5 is dead cane.
Cane ratings are of the form [# of canes]as[rating].

Overall success in inoculating canes in transgenic and non-transgenic vines was very high. In some cases the tags marking inoculated canes in HA-transgenic vines were missing so no rating was made. 0 ratings of canes on HA-transgenic canes occurred on vines where at least 2 of the other canes on that vine expressed some PD symptoms; thus we believe the inoculum that was used to inoculate 0 scoring canes was viable. However, it is certainly possible that the inoculum was not taken into actively transpiring xylem vessels which could result in an unsuccessful inoculation.
Overall PD symptoms severity was higher in the non-transgenic positive controls than in the HA-transgenic vines; results that were similar to what we observed in the greenhouse inoculations. It is also clear from the field inoculations that none of the transgenic lines completely prevented the onset of PD symptoms in inoculated canes, again results that were observed in greenhouse trials.

Objective 3:
Canes were cut back to 2 buds once vines were completely dormant in January/February 2015. The vines were rated for PD symptoms in late August 2015. 95% of the inoculated canes had some level of leaf scorching which indicated our inoculation procedure was successful as shown in figures 3A 3B,and #C.

Figure 3A: Dead Adhesion domain vine that collapsed in June 2015

Figure 3B: Full length transgenic cane rated #2 in August 2015

Figure 3C: Healthy appearing full length transgenic vine, August 2015

SUMMARY OF ACCOMPLISHMENTS

Eight HA-transgenic lines were shown by qRT-PCR to express HA mRNA. Greenhouse inoculations of the 8 HA-transgenic Thompson seedless grapes with cultured Xf cell showed all lines expressed less severe symptoms of PD than inoculated, non-transgenic controls. All transgenic lines as well as non-transgenic Thompson seedless vines that used as positive and negative controls were planted in the field in spring 2013; the vines grew well and were trained as bilateral cordons. Four shoots on each vine were mechanically inoculated with wt Xf in May 2014. PD disease symptoms on inoculated and non-inoculated shoots were evaluated in September 2014. A high percentage of the inoculated shoots developed scorch leaves typical of PD symptoms indicating our needle inoculation technique was successful. PD symptoms severity ratings were lower among HA-transgenic lines than inoculated non-transgenic grapevine controls. Canes from transgenic and non-transgenic vines were collected to determine Xf titers by qPCR. All shoots were pruned back to 2 buds in January/February 2015 and allowed to push during the 2015 growing season. Spring shoot growth and PD symptoms were recorded in September 2015 to determine if the Xf infections overwintered and formed systemically infected vines. Most of the adhesion domain vines and full length HA gene transformants had some vines that appeared PD free. However with other reps of the transgenic lines some reps were either dead or had PD symptoms on inoculated canes that varied in severity.

If Xf populations in HA-transgenic lines are low enough to prevent fruit symptoms and vine dieback we may have produced transgenic vines that are functionally tolerant of Xf infection. Their possible use as rootstocks grafted with non-transgenic scions will be evaluated in the coming years.

RESEARCH RELEVANCE

Previous research in Dr. Kirkpatrick's lab identified two hypervirulent mutants of *Xylella fastidiosa* (Xf). These mutations were in large hemagglutinin (HA) adhesion genes called *Hfx*A and *Hfx*B. *Hxf* mutants also showed a marked decrease in cell-cell clumping when grown in liquid culture. He hypothesized that if Hxf protein, or a portion of the Hxf protein that mediates adhesion, could be expressed in the xylem fluid of transgenic grapevines then, perhaps, *Xf* cells would clump together at the site of insect delivery and be less capable of colonizing grapevines. Transgenic HA-expressing grapevine line were produced, and mechanical inoculated with Xf cells in the greenhouse. These transgenic lines were transplanted to the APHIS permitted field site for transgenic plants coordinated by Dr. Gilchrist in Solano County in spring 2013. These vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines. The shoots cut back to 2 buds and then mechanically inoculated 4 shoots/vine with a mixture of Temecula and Stag's Leap Xf strains in April 2014. Over 95% of the inoculated canes showed scorch symptoms typical of PD in September 2014 indicating that the inoculations were successful with at least two PD-symptomatic canes were present on all inoculated vines. In January, 2015 the shoots were trimmed to two buds and the emerging shoots and the entire vine were rated for PD symptoms in August 2015. In 3 out of the 5 lines expressing the HA adhesion domain the majority of the vines showed no PD symptoms, however PD symptoms were evident on the canes other adhesion domain transgenes. These are initially encouraging results that confirm the results of greenhouse testing.

LAYPERSON SUMMARY

Dr. Kirkpatrick invested more than 10 years investigating the role of hemagglutinins (HA), large proteins that mediate the attachment of bacteria to themselves and to various substrates, how these proteins may play in Pierce's disease pathogenicity and insect transmission. Early work showed that HA mutants were hypervirulent: they caused more severe symptoms and killed vines faster that vines inoculated with wild type (wt) *Xylella fastidiosa* (Xf) cells (Guilhabert and Kirkpatrick, 2005). HA mutants no longer clumped together in liquid cultures like wt cells, indicating that cell adhesion molecules were important in establishing a pathogenic population of bacteria in the grape xylem. The current project is testing the hypothesis that HAs expressed in xylem sap of transgenic grapevines may act as a "molecular glue" that would aggregate and thus slow the movement of Xf cells introduced into grapevines. Transgenic lines expressing various HA constructs were moved to the field in spring 2013. The vines grew well and were trained up to the wire and established as a conventional bilateral cordon vines with 2 bud spurs and then inoculated 4 shoots/vine with the Fetzer strain of Xf in April 2014. PD symptoms were rated in September 2014 on the inoculated shoots, including whether the bacteria had moved to adjacent non-inoculated shoots and were expressing PD symptoms. Over 90% of the inoculated canes showed scorch symptoms typical of PD in September 2014 indicating that the inoculations were successful. In only 1 instance were PD symptoms observed on an adjacent, un-inoculated shoot. The summary observation is that PD symptom severity was lower in the inoculated HA-transgenic grapevines than the Xf-inoculated non-transgenic controls. The pruning and inoculation programs was repeated beginning in January, 2015 and the shoots were rated for PD symptoms in August 2015.The results continue to be encouraging in three out of the 5 independently transformed lines expressing the HA adhesion domain wherein the majority of the vines showed no PD symptoms. In the 3 full length HA gene construct lines the majority of all the vines were healthy with no PD symptoms. These, initially encouraging results, are consistent with the results of greenhouse testing. The canes will be cut back to 2 bud spurs in the spring of 2016 and evaluated for PD symptoms as the shoots emerged.

STATUS OF FUNDS. All funds budget for these projects will be expended at the end of the current funding cycle as proposed.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY. The grape plants containing the anti-PCD genes and the grafted rootstocks will require the use of several patented enabling

technologies. Record of invention disclosures have been submitted to the UC Office of Technology Transfer. The research proposed reported herein will provide data on the activity and mechanism of action of the protective transgenes in grape relative to the presence, amount and movement of Xylella fastidiosa in the transformed and untransformed grape plants.

LITERATURE CITED:

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